Biochemistry

 Volume 21, Number 6 March 16, 1982

Conformational and Functional Changes in the Fourth Component of Human Complement Produced by Nucleophilic Modification and by Proteolysis with $C1\bar{s}$[†]

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ABSTRACT: Fluid-phase hemolytic inactivation of C4 by specific proteolytic cleavage ($C1\bar{s}$) or by nucleophilic modification (with methylamine or hydrazine) in the absence of proteolysis leads to the same conformational end state in the molecule, as judged by a number of spectroscopic criteria. Since both inactivation processes in C4 are thought to result in the scission of an internal thioester bond in the α chain of the molecule [Janatova, J., & Tack, B. F. (1981) *Biochemistry 20*, 2394–2402], a crucial role for this cyclic structure in the determination of the three-dimensional folding of the molecule is strongly implied. Correlating with the loss of the thioester bond, one can demonstrate substantial alterations in the local environments of aromatic chromophores within the molecule by near-UV circular dichroism and by UV absorption difference spectroscopy. A small change in the backbone conformation was also apparent (far-UV circular dichroism) as was a decrease in the surface hydrophobicity (8-anilino-1-naphthalenesulfonate fluorescence) following cleavage of the thioester bond. Further evidence for the virtually complete conformational identity between C4b and the methylamine-modified protein [$C4(CH_3NH_2)$] comes from functional assays showing that both molecules can interact with C2 and also display a similar C4 binding protein dependent susceptibility to cleavage by the C3b inactivator enzyme. The kinetics of the nucleophile-induced conformational change were relatively slow and were not limited by the rate of the chemical modification event. In contrast, the rate of the conformational transition in the proteolytically activated molecule appeared to be dependent on the rate of enzymatic cleavage per se. When monitored by two different spectral probes, the kinetics of the conformational changes in $C4(CH_3NH_2)$ were not superimposable, suggesting a sequential folding rearrangement in different portions of the molecule. The kinetics of functional site acquisition in $C4(CH_3NH_2)$ were also found to be slower than the rate of chemical modification and thus appeared to be dependent on the acquisition of a C4b-like conformation. However, it was found that the molecule acquired the ability to bind C2 significantly sooner than it was able to interact with C4 binding protein. Thus, in addition to corroborating the kinetic complexity of the conformational changes observed spectroscopically, these observations strongly suggest that the binding sites for C2 and C4 binding protein in C4b are topographically distinct.

The fourth component of complement, C4, circulates in plasma as a functionally inactive precursor molecule. It consists of three nonidentical disulfide-linked chains, designated α, β, and γ, having molecular weights of 93 000, 78 000, and 33 000, respectively (Schreiber & Müller-Eberhard, 1974). C4 is converted into its functionally active form when $C1\bar{s}$, generated through the interaction of C1 with an immune complex or other activator of the classical pathway, cleaves the peptide bond between residues 77 and 78 of the α chain to produce an activation peptide, C4a (M_r 9000), and a major cleavage fragment, C4b (M_r 186 000) (Budzko & Müller-Eberhard, 1970). Structural changes produced by this cleavage result in the acquisition by C4b of stable interaction sites for the protein ligands C2 (Müller-Eberhard et al., 1967) and C4 binding protein (C4-bp)[1] (Gigli et al., 1979), these two complement components being respectively involved in the assembly and regulation of the classical pathway C3 convertase. C4b is itself a ligand for the CR-I immune adherence receptor present on a variety of circulating cell types (Bianco & Nussenzweig, 1977), and C4a has recently been shown to be an anaphylatoxin (Gorski et al., 1979). In addition to these stable functional sites, nascent C4b possesses the transient ability to bind firmly to the surface of the activating substance (Müller-Eberhard & Lepow, 1965). The chemical nature of this labile surface attachment site in C4b and in the structurally related protein C3b has recently been substantially clarified. Law and co-workers have shown that nascent C3b

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[1] Abbreviations: C3bINA, C3b inactivator; C4-bp, C4 binding protein; $C4(CH_3NH_2)$, C4 modified by incorporation of methylamine; CR-I, complement receptor I; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ANS, 8-anilino-1-naphthalenesulfonate; PBS, phosphate-buffered saline; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism; E_a, activation energy.

0006-2960/82/0421-1109$01.25/0

(Law & Levine, 1977; Law et al., 1979) and C4b (Law et al., 1980a) bind covalently to the target surface and that this attachment occurs via an ester linkage. Evidence for an amide linkage has also been reported (Campbell et al., 1980; Sim et al., 1981). In either case, the complement component is the acyl donor, while the receptive surface provides the attacking nucleophile. Much information about the chemical nature of the reactive carbonyl in C3 and C4 has recently been derived from studies on the mechanism through which nitrogen nucleophiles hemolytically inactivate these molecules. The major conclusions of these studies may be summarized as follows. (1) Hemolytic inactivation correlates with covalent incorporation of 1 mol of nucleophile per mol of C3 or C4 (Tack et al., 1980; Pangburn & Müller-Eberhard, 1980; Law et al., 1980b; Howard, 1980; Gorski & Howard, 1980; Lundwall et al., 1981; Sim et al., 1981). (2) Nucleophilic modification leads to the liberation of a free sulfhydryl group but does not result in the cleavage of any peptide bonds (Janatova et al., 1980; Janatova & Tack, 1981; Pangburn & Müller-Eberhard, 1980). (3) Both the liberated sulfhydryl group and the nucleophile-modified residue have been localized to the C3d region of C3 (Tack et al., 1980), and indirect evidence suggests that these residues are located within the C4d region of C4 (Janatova & Tack, 1981). In the case of C3, sequence analysis has shown that they originate from a cysteinyl sulfhydryl and a glutamic acid side chain located only two residues apart in the primary structure of the α chain. (4) Hemolytic inactivation with concomitant sulfhydryl exposure in C3 and C4 can also be produced by low concentrations of chaotropes or by a slow freezing and thawing process (Janatova et al., 1980; Isenman et al., 1981; Von Zabern et al., 1981). In these cases, it is assumed that water acts as the nucleophile. These observations have led to the postulation of an internal thioester in native C3 and C4 comprised of the reactive cysteine and glutamic acid residues (Tack et al., 1980; Pangburn & Müller-Eberhard, 1980; Law et al., 1980b; Sim et al., 1981). Proteolytic activation of C3 and C4 in some way renders the carbonyl portion of the thioester more susceptible to attack either by nucleophiles on a receptive surface or by the solvent. While the former process results in a covalent attachment via transacylation, solvolysis of the thioester results in the production of a hemolytically inactive molecule in the fluid phase.

Pangburn & Müller-Eberhard (1980) made the observation that C3 which lacked the thioester bond possessed C3b-like functional activity. Through the use of a number of spectral probes, this C3b-like activity for the thioester-cleaved protein could be readily understood in terms of the similar conformational states of C3b and the nucleophile-modified protein (Isenman & Cooper, 1981; Isenman et al., 1981). The apparent correlation between cleavage of the thioester bond in the C3 α chain and the acquisition of a conformation capable of expressing functional site activity prompted a similar investigation in the structurally related C4 molecule. Accordingly, the nature and extent of the conformational changes in C4 brought about by proteolytic activation were examined and compared to those produced by nucleophilic modification. Our findings indicate that, like C3, the conformational state of C4 is primarily dictated by the presence or absence of the internal thioester bond. We further show that following nucleophilic scission of the thioester bond in C4, a relatively slow and kinetically complex conformational change ensues with the concomitant appearance of C4b-like functional properties.

Materials and Methods

Chemicals and Reagents. Lactoperoxidase, bovine trypsin, soybean trypsin inhibitor, methylamine hydrochloride, and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Sigma, St. Louis, MO. Hydrazine and 8-anilino-1-naphthalenesulfonate were purchased from Eastman, Rochester, NY, and ^{125}I-labeled sodium iodide was obtained from Amersham, Oakville, Ontario.

Purified Complement Components. Fresh frozen human plasma, generously provided by the Toronto Western Hospital blood bank, was the source material for the preparation of all complement components. C4 was prepared by a modification (Gorski et al., 1981) of the procedure of Bolotin et al. (1977). C2 (Nagasawa & Stroud, 1977), C3bINA (Nagasawa et al., 1980), C4 binding protein (Nagasawa & Stroud, 1980), and $C1\bar{s}$ (Valet & Cooper, 1974) were prepared as described previously. C4 was converted to C4b with $C1\bar{s}$ (1% w/w) at 37 °C for 1 h, and the extent of conversion was assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis run according to the method of Fairbanks et al. (1971) in 5% gels. Protein iodination with ^{125}I was performed by using the lactoperoxidase procedure described by Marchalonis (1969) except that the hydrogen peroxide stock solution was 2.2 mM. The presence of free sulfhydryl groups in C4 or its derivatives was quantitatively assessed by using the Ellman reagent (DTNB) as described by Janatova et al. (1980).

Buffers. The following veronal–NaCl buffers were used (Rapp & Borsos, 1963): VB, veronal-buffered saline, pH 7.3, containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (μ = 0.15); GVB, VB containing 0.1% gelatin; SGVB, low ionic strength (μ = 0.06) VB made isotonic with sucrose and containing 0.1% gelatin; SGVB-Mg^{2+}, SGVB in which the Mg^{2+} concentration has been increased to 10 mM; GVBE, GVB made 10 mM in EDTA. Phosphate-buffered saline (PBS) consisted of 0.01 M sodium phosphate–0.15 M NaCl, pH 7.4. In some experiments, the pH of this buffer was adjusted to 8.0 with sodium hydroxide.

Nucleophilic Modification of C4. C4 was treated with 20 mM hydrazine or 20 mM methylamine at pH 8.0 for 4 h at 37 °C. Excess reagents were removed by dialysis into PBS, pH 7.4.

Hemolytic Assays. Sheep erythrocytes sensitized with IgM antibody (EA) or IgM antibody and C4b (EAC4) were prepared according to standard techniques (Rapp & Borsos, 1970), except that heated human serum (56 °C, 30 min) was used as the source of C4. C4 hemolytic activity was assessed in a one-step assay by using EA cells and a C4-deficient human serum essentially as described by Gaither et al. (1974) for C4-deficient guinea pig serum. The time course of C4 hemolytic inactivation at various temperatures by methylamine or hydrazine (20 mM, pH 8.0) was followed by measuring the residual C4 hemolytic activity of time-point aliquots diluted 200-fold into ice-cold GVB. Results were calculated on the basis of the "one-hit" hypothesis (Rapp & Borsos, 1970) and were expressed as a percentage of the C4 activity present at time zero.

The ability of native C4, C4b, or nucleophile-modified C4 to bind C2 in the fluid phase was assessed by using a C2-dependent hemolysis inhibition assay. Briefly, 0.1 mL of iodine-oxidized human C2 (Polley & Müller-Eberhard, 1967) in SGVB-Mg^{2+} at a concentration sufficient to give approximately 80% hemolysis in the absence of inhibitor protein was added to 0.1-mL serial 2-fold dilutions of the test protein in the same buffer at 0 °C. EAC4 cells (1.5×10^7) were added, and the tubes were allowed to stand on ice for 5 min, at which time the cells were washed with 5 mL of ice-cold GVB-Mg^{2+}. The cells were then resuspended in 0.2 mL of this buffer and treated with excess human euglobulin $C\bar{1}$ reagent for 10 min

at 30 °C. Finally, hemolysis was developed at 37 °C by the addition of 1 mL of 1:50 guinea pig serum in GVBE (C-EDTA), and the absorbance at 412 nm of the supernatants was read. Results were calculated in terms of the one-hit hypothesis and were expressed as a percentage of the activity observed in the absence of inhibitor protein. When this assay was used to follow the kinetics of the acquisition of C2 binding activity, time-point aliquots of a C4 plus methylamine incubation mixture (0.3 mg/mL C4, 20 mM methylamine, 37 °C, pH 8.0) were diluted 250-fold into ice-cold SGVB-Mg^{2+}, and 0.1 mL of this solution was then added to a tube containing 0.1 mL of C2 at a concentration sufficient to give 80% hemolysis in the absence of inhibitor. After 30 s, EAC4 cells (1.5×10^7) were added, and the incubation was continued for a further 5 min at 0 °C. The cells were washed once with 5 mL of cold SGVB-Mg^{2+}, and the pelleted cells were stored on ice until the end of the time course (45 min), at which time all of the kinetic aliquots were treated with $C\bar{1}$ and C-EDTA as described above. In control experiments, it was found that the activity of the washed EAC4b2 cells was stable on ice for the duration of the experiment.

The effective molecule titration of C3 in human serum made 10 mM in EGTA and 5 mM in Mg^{2+} was performed with $EAC4^{oxy}2$ cells, as described previously (Cooper & Müller-Eberhard, 1970).

Cleavage of C4b or C4(CH_3NH_2) by C3bINA and C4-bp. A mixture consisting of 10 μg of C4-bp, 2 μg of C3bINA, and 10 μg of radiolabeled C4, C4b, or C4(CH_3NH_2) was incubated at 37 °C for 5 min, at which time the enzyme was inactivated by the addition of $NaDodSO_4$ sample buffer followed immediately by boiling for 30 s. The extent of cleavage into C4d and C4c (or C4c-like) fragments was determined from the radioactive profiles of $NaDodSO_4$–polyacrylamide gels run under nonreducing conditions.

Spectral Measurements. Using the procedures described previously (Isenman & Cooper, 1981; Isenman et al., 1981), we measured circular dichroism (CD), fluorescence, and ultraviolet difference absorption by using, respectively, a Jasco J-41 spectropolarimeter (Japan Spectroscopic, Tokyo), an Aminco SPF500 spectrofluorometer (American Instruments, Silver Springs, MD), and a Cary 219 spectrophotometer (Varian Instruments, Palo Alto, CA). Spectra and base lines were digitized and stored on magnetic tape by using a time base data logger built by the Division of Medical Computing at the University of Toronto. A typical spectrum or base line would consist of 300–500 points. Computerized processing and plotting of the data included a smoothing routine consisting of a single pass of a 19-point least-squares procedure as described by Savitzky & Golay (1964). Except where indicated otherwise, spectral measurements were made at 22 °C. UV absorption differences spectral changes in C4 induced by methylamine (which itself did not have an absorption spectrum over the wavelength range of interest, 330–240 nm) were obtained by using a matched pair of self-masking semimicro quartz cuvettes (Hellma). Each cell contained 0.9 mL of C4 (1 mg/mL), and following the establishment of a protein–protein base line, 9 μL of 2 M methylamine was added to the sample cuvette while 9 μL of solvent was added to the reference cuvette. The conformational change induced was allowed to go to completion (in the absence of continuous irradiation), and the difference spectrum was recorded. Since $C1\bar{s}$ itself has an absorption spectrum, the difference spectrum produced by the action of $C1\bar{s}$ on C4 required the use of a matched pair of rectangular tandem cells (Hellma). One side of each tandem cell contained 0.9 mL of C4 (1.2 mg/mL)

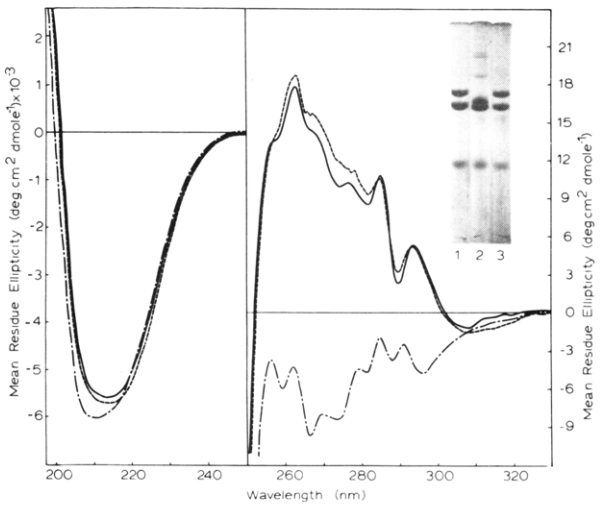

FIGURE 1: Near- and far-ultraviolet circular dichroism spectra of native C4 (–·–), C4 treated with $C1\bar{s}$ (—), and C4 nucleophile modified with methylamine (---). Inset: Electrophoretic analysis of the proteins whose spectra were recorded on $NaDodSO_4$–polyacrylamide gels run under reducing conditions. (1) Native C4; (2) C4 + $C1\bar{s}$; (3) C4(CH_3NH_2).

while the other side contained a dilute solution of $C1\bar{s}$ (0.012 mg/mL). A premixing base line was obtained, and then the conversion of C4 to a C4a + C4b mixture was initiated in the sample cuvette by repeated inversion of the cell. The difference spectrum was then recorded.

The concentration of C4 was determined spectrophotometrically at 280 nm by using 8.2 as the value for $E^{1\%}_{280nm}$. The extinction coefficient was determined from UV absorbance measurements and a concentration determination by using a Hilger and Watts Rayleigh interference refractometer (Tang & Adams, 1973; Babul & Stellwagen, 1969). The value obtained agrees well with the extinction coefficient of C4 reported previously by Nagasawa & Stroud (1977).

Kinetic CD and Fluorescence Measurements. For kinetic experiments in which the spectral change at a single wavelength was continuously monitored, the analog output of the instruments was digitized and stored on magnetic tape by using the data logger referred to above. Analyses of the kinetic data in terms of theoretical models were performed by iteratively fitting the data to curves described by the appropriate differential rate equations. The procedures employed have been described by us previously (Isenman et al., 1981).

Results and Discussion

Equilibrium Studies. (1) Circular Dichroism. The circular dichroism spectra (330–198 nm) of native C4, C4 converted to an equimolar C4a + C4b mixture with $C1\bar{s}$, and C4 modified with methylamine, C4(CH_3NH_2), are shown in Figure 1. Also shown in the inset of this figure are the $NaDodSO_4$–polyacrylamide gel analyses of the proteins whose spectra were recorded. In addition, the free sulfhydryl content of these proteins was assessed and found to be less than 0.2 mol/mol for the native protein, while approaching 1 mol/mol for both C4(CH_3NH_2) and the C4a + C4b mixture. The CD spectra may be divided into two parts, the near-UV portion (330–250 nm), which reflects tertiary and quaternary folding as it is sensitive to the local asymmetric environment of aromatic chromophores in the molecule, and the far-UV region (250–198 nm), which reports primarily on secondary structure. The native protein exhibits a complex series of negative CD transitions in the near-UV region of the spectrum. Proteolytic activation by $C1\bar{s}$ leads to very pronounced positive shifts over the entire near-UV spectral range, indicating substantial

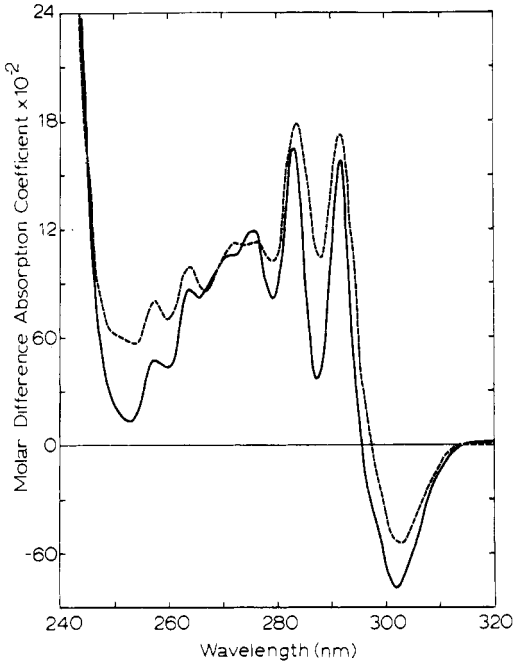

FIGURE 2: Ultraviolet absorption difference spectra of C4 modified with 20 mM methylamine (—) and of the C4a + C4b digestion mixture produced by C1s proteolysis (- - -) vs. the native protein in each case.

changes in the local environments of a number of aromatic amino acid side chains. It can be seen that a very similar CD spectrum is obtained for the methylamine-modified protein, and an identical spectrum was also observed for C4 modified with hydrazine. C4 which had lost approximately 50% of its hemolytic activity as a result of freezing and thawing displayed the same spectral shifts relative to those of the native protein, although in this case the magnitude of the change was proportionately smaller (data not shown).

In the far-UV region, native C4 displays a single asymmetrical minimum at 209 nm (−6000 deg·cm^2 dmol^{-1}), and the spectrum shows a change in its polarity at 199.5 nm. Conversion of C4 to an equimolar mixture of C4a + C4b with C1s, or nucleophilic modification with methylamine or hydrazine, results in a small decrease in the strength of the CD band, but more notably, the shape of the band is broadened such that the minimum now occurs at 213 nm, and the change in polarity is red shifted to 201 nm. Thus, unlike C3, where proteolytic conversion or nucleophilic modification appeared to have no significant effect on secondary structure, the changes observed in the far-UV CD spectrum of C4 provide evidence of some degree of alteration in the backbone conformation accompanying these treatments. Qualitatively similar observations regarding changes in the shape of the far-UV CD spectrum of C4 following proteolytic conversion to C4b have recently been reported by Gorski et al. (1981). In contrast to their data, however, we consistently find that fluid-phase inactivation of C4, by either nucleophiles or C1s, results in a decrease in the intensity of the far-UV CD spectrum.

(*2*) *UV Absorption Difference Spectroscopy.* The UV absorption difference spectra produced by proteolytic conversion of C4 with C1s or by nucleophilic modification are shown in Figure 2. The similarity of these two spectra provides further evidence that the same conformational changes in C4 are produced by either process leading to fluid-phase hemolytic inactivation. The most striking feature of these difference spectra is that they are dominated by tryptophan transitions

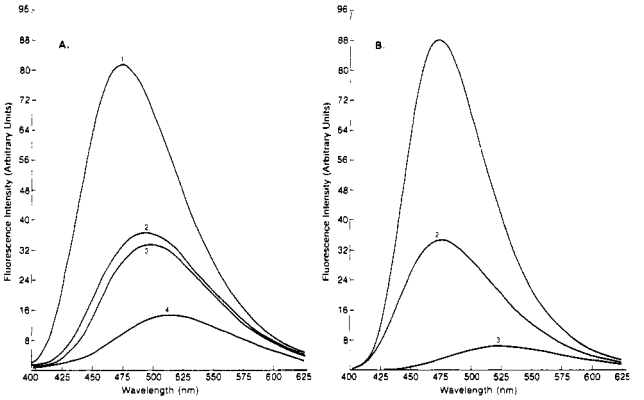

FIGURE 3: (A) Fluorescence emission spectra of 2 × 10^{-5} M ANS in the presence of native C4 (1); C4 nucleophile modified with 20 mM methylamine (2); C4 converted to a C4a + C4b mixture with C1s (3); PBS buffer (4). The protein concentration was 2.2 × 10^{-6} M in each case. (B) Fluorescence emission spectra of 2 × 10^{-5} M ANS in the presence of C3b [produced from C3 by limited trypsinization (1% w/w, 2 min, 22 °C)] (1); native C4 (2); PBS buffer (3). The protein concentration was 2.04 × 10^{-6} M for both C3b and C4. In all emission spectra, excitation was at 386 nm, and 5-nm band-pass excitation and emission slits were used. All spectra have also been corrected for a small contribution due to buffer scattering.

occurring at 291 and 283 nm while at 286 nm, where perturbed phenolic side chains of tyrosine give rise to a spectral band, the difference spectra display a minimum. The positive direction and the magnitude of the molar difference absorption coefficients of 1575 and 1700 for C4(CH_3NH_2) and C4 treated with C1s, respectively, closely approach the expected value of 1600 for the net sequestration from the solvent of one tryptophan side chain as a result of the conformational change (Donovan, 1969). The negative band at 301 nm is thought to arise from a charge perturbation of indole ring electronic transitions (Ananthanarayanan & Bigelow, 1969a,b). Below 270 nm, there exist a series of positive transitions which likely arise, at least in part, from phenylalanine residues which have become buried as a result of the conformational change accompanying the cleavage of the internal thioester bond.

(*3*) *Fluorescence Measurements.* Employing the fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS), we have previously reported that both enzymatic conversion of C3 to C3b and nucleophilic modification with methylamine result in an increase in the surface hydrophobicity of the molecule as evidenced by the very pronounced enhancement of ANS fluorescence (Isenman et al., 1981; Isenman & Cooper, 1981). In experiments to be presented elsewhere, we have found that the ANS fluorescence enhancement produced by C3b is sensitive to the presence of the C3b ligands, factor B and β1H. These finding suggested that the increase in surface hydrophobicity detected by ANS in C3b or C3(CH_3NH_2) reflected an exposure to the solvent of regions involved in the binding sites for these two proteins. Since C4 also acquires ligand binding properties following enzymatic conversion to C4b, it was surprising to find that this treatment led to an approximately 2.5-fold decrease in ANS fluorescence and a 20-nm red shift of the maximum (473 to 493 nm) relative to that of the native protein (Figure 3A). A similar change is also produced when C4 is modified with methylamine (Figure 3A). The ANS fluorescence intensity at 473 nm for even native C4 is about 2.6-fold weaker than that of C3b when compared at the same molar concentrations of protein and ANS (Figure 3B). From these findings, one might speculate that the forces involved in the binding of C2 or C4-bp to C4b are different from those involved in the interaction between C3b and factor B or β1H.

Table I: Kinetic Constants for the Rate of Hemolytic Inactivation of C4 and C3 by Methylamine and Hydrazine

protein	temp (°C)	pH	nucleophile	pK_a[a]	concn of free base (M)	pseudo-first-order rate constant (s^{-1})	pseudo-first-order $t_{1/2}$[b] (min)	calcd second-order[c] rate constant (M^{-1} s^{-1})	E_a (kcal/mol)
C4	22	8.0	20 mM hydrazine	8.05	9.46×10^{-3}	1.05×10^{-3}	10.9	1.11×10^{-1}	
	30	8.0	20 mM hydrazine	7.84	11.82×10^{-3}	2.82×10^{-3}	4.1	2.39×10^{-1}	16.4
	37	8.0	20 mM hydrazine	7.67	13.66×10^{-3}	5.88×10^{-3}	1.96	4.31×10^{-1}	
C4	22	8.0	20 mM methylamine	10.69	40.75×10^{-6}	4.98×10^{-4}	23.1	12.22	
	30	8.0	20 mM methylamine	10.43	74.03×10^{-6}	1.81×10^{-3}	6.3	24.45	16.5
	37	8.0	20 mM methylamine	10.21	121.45×10^{-6}	5.81×10^{-3}	1.98	47.84	
C3[d]	23.5	8.0	50 mM methylamine	10.64	114.3×10^{-6}	4.3×10^{-4}	26.7	3.76	
	30	7.9	50 mM methylamine	10.43	147.1×10^{-6}	8.57×10^{-4}	13.4	5.83	14.5
	37	7.8	50 mM methylamine	10.21	192.0×10^{-6}	2.11×10^{-3}	5.45	10.99	

[a] The pK_a value at a given temperature for each nucleophile was calculated from the tabulated values of pK_a and heat of proton ionization at 25 °C (Sober, 1968) by using the van't Hoff relationship. [b] $t_{1/2}$ is defined as (ln 2)/k for the first-order or pseudo-first-order reactions. [c] Calculated by dividing the pseudo-first-order rate constant by the molar concentration of the nucleophile in the free base form. These latter values were arrived at by using the temperature-corrected pK_a values in the Henderson-Hasselbalch equation. [d] The pseudo-first-order rate data for C3 are reproduced from Isenman et al. (1981).

The intrinsic fluorescence spectra of C4, C4 treated with $C1\bar{s}$, and C4(CH_3NH_2) were also examined. Relative to the native protein, the two hemolytically inactive forms display approximately 5% less intensity of intrinsic fluorescence (excitation at 280 nm), and the emission maximum which is at 332 nm in the native protein is blue shifted 1 nm in the hemolytically inactivated proteins (data not shown). The small effect on intrinsic fluorescence in C4 is thus very similar to that observed when C3 is enzymatically converted to C3b (Isenman & Cooper, 1981) and in both proteins probably reflects the perturbation of a rather limited number of tryptophan residues in the conformation adopted by the thioester-cleaved molecules.

Kinetic Studies. (*1*) *Rate of Nucleophilic Modification.* The correlation between the loss of hemolytic activity, the covalent incorporation of the nucleophile, and the appearance of a free SH group in C4 has been well documented (Janatova & Tack, 1981). We have therefore monitored the kinetics of the nucleophilic modification in C4 through the loss of its hemolytic activity. The inactivation was carried out under pseudo-first-order conditions at temperatures of 22, 30, and 37 °C by using either 20 mM hydrazine or 20 mM methylamine as the nucleophile. The results of these experiments are shown in Table I. Based on total nucleophile concentration, it can be seen that at room temperature hydrazine is a more effective nucleophile than is methylamine, the half-time of inactivation being approximately 11 min for the former and 23 min for the latter. At 37 °C, however, the rates of inactivation become indistinguishable, being 2 min in each case. The pseudo-first-order rate constants were transformed into bimolecular rate constants at each temperature by dividing by the concentration of the nucleophile in the free base form. This procedure assumes that the reaction is first order with respect to the nucleophile, a fact which has been recently demonstrated by Law et al. (1980b). The bimolecular rate constant governing inactivation of C4 by methylamine is approximately 4-fold greater than the equivalent rate constant for C3 inactivation by this nucleophile (Isenman et al., 1981) over the temperature range of 22–37 °C (Table I). The rate constants shown in Table I were used to construct Arrhenius plots, and in each case, a linear relationship was obtained. The activation energies for nucleophilic attack on C4 were calculated to be 16.4 and 16.5 kcal/mol for hydrazine and methylamine, respectively. A comparable value of 14.5 kcal/mol was obtained as the activation energy of thioester cleavage in C3 by methylamine.[2] Thus, the greater sensitivity of C4 to hemolytic inactivation by methylamine cannot be explained simply on the basis of a significantly lower activation energy barrier for nucleophilic attack. It may, however, reflect a difference in the accessibility to the reagents of the thioester bond in the two molecules.

(*2*) *Kinetics of the Conformational Change Accompanying Thioester Cleavage in C4.* When C4 was proteolytically activated with $C1\bar{s}$, the rate of the spectroscopic change was dependent on the amount and activity of the enzyme employed, and thus it did not appear to be rate limited by cleavage-induced unimolecular transitions. Having established the pseudo-first-order rate constant for nucleophilic scission of the internal thioester of C4 under a specified set of conditions, we wished to determine whether the kinetics of the accompanying conformational changes were reflective solely of the bimolecular chemical modification event, or whether they were rate limited by subsequent unimolecular processes. Accordingly, the time course of the spectral change in the near-UV CD at 262 nm and in ANS fluorescence emission at 473 nm was continuously monitored following the addition of methylamine to native C4 (37 °C, 20 mM methylamine, pH 8.0). These experiments are shown in Figure 4. Using either monitoring system, it is clear that the rate of the nucleophile-induced conformational change was much slower than the rate of thioester cleavage. The kinetics observed using the CD change as the probe consistently showed a 2–3-min lag phase, while in the ANS fluorescence monitoring system a much longer lag phase of 7–8 min was observed. It can also be seen that, whereas the overall spectral change is 50% complete in about 15 min (900 s) in the CD monitoring system, the equivalent point in the ANS system requires an additional 6 min (i.e., 1260 s).

The relative rapidity of the conformational change induced by proteolytic activation compared to that observed in the thioester-cleaved, but peptide bond intact, molecule suggests a role for C4a and/or its linkage peptide bond (between residues 77 and 78 of the α chain) in the kinetics of the conformational processes. In order to further demonstrate this

[2] We have previously reported a value of 21.4 kcal/mol as the activation energy for the nucleophilic modification of C3 by methylamine (Isenman et al., 1981). The second-order rate constants used in this original calculation were based on total methylamine concentration and did not take into account the effect of temperature on the pK_a of the nucleophile. The corrections for the temperature dependence of the pK_a of methylamine and the resulting effect on the concentration of the free base form of the nucleophile are outlined in Table I.

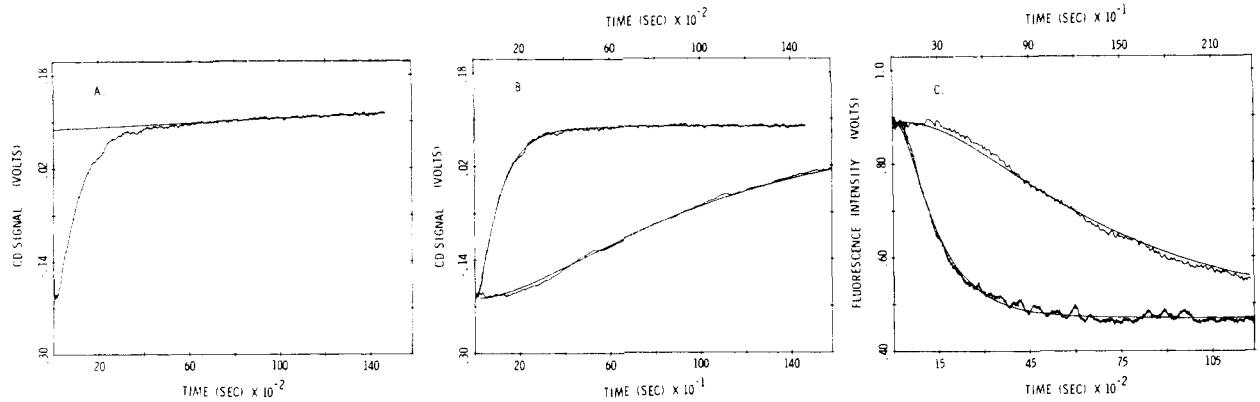

FIGURE 4: Kinetics of the spectroscopic change induced by the addition of methylamine to native C4 (20 mM methylamine, 37 °C, pH 8.0). (A) Continuous monitoring of the conformational transition as probed by the change in the near-UV CD signal at 262 nm. The experiment was done at a protein concentration of 1.5 mg/mL in a 1-cm cell with a full-scale expansion of 5 mdeg. The units of the ordinate are derived from the digitization process where 1 V is equal to 8.06 mdeg of ellipticity. The sampling interval was 1.5 s for the first 150 s and 15 s thereafter. The straight line drawn by linear regression analysis through the points onward of 10 000 s was used as a sloping base line in obtaining the corrected data set shown in panel B (see text for further details). (B) The upper curve (and time scale) shows the corrected version of the data from panel A. The lower curve (and time scale) represents an expansion of the first 10% of the recorded data. The noisy curves depict the raw data, while the smooth curves were calculated from the fit parameters derived from a numerical treatment of the data in terms of mechanistic Scheme II. (C) Continuous monitoring of the conformational transition as probed by the change in the ANS fluorescence emission at 473 nm (386-nm excitation). The lower curve (and lower time scale) shows the complete data set while the upper curve (and time scale) shows an expansion of the first 20% of the collected data. The sampling interval was 1.0 s for the first 100 s and 10 s thereafter. The smooth curves were calculated from the mechanistic analysis of the data in terms of Scheme I. Protein and ANS concentrations were 2.6×10^{-6} and 2.0×10^{-5} M, respectively. A very narrow excitation slit was used (1-nm band pass) to minimize long-term irradiation artifacts. Even with a 10-nm band-pass collection slit, the noise level remained substantial.

Table II: Rate Constants Derived from a Mechanistic Analysis of the Spectral Changes in C4 Treated with Methylamine

signal monitored	mechanistic scheme	k_0 (s^{-1})	k_1 (s^{-1})	k_2 (s^{-1})	ΔV_1 ($V M^{-1}$)	ΔV_2 ($V M^{-1}$)
CD, 262 nm	II	3.7×10^{-3} (3.1) [a]	1.1×10^{-3} (10.4)		4.2×10^{4}	
SD [b]		6.7×10^{-5}	6.5×10^{-6}		4.3×10^{1}	
ANS fluorescence, 473 nm	I	4.6×10^{-3} (2.5)	4.6×10^{-3} (2.5)	8.3×10^{-4} (13.9)	-0.6	-2.05×10^{5}
SD		1.1×10^{-2}	1.1×10^{-2}	7.4×10^{-6}	5.5×10^{2}	4.2×10^{2}

[a] The numbers in parentheses are the $t_{1/2}$ values, in units of minutes, for the various kinetic steps of the mechanistic scheme. [b] SD, the standard deviation, refers to the error in the kinetic parameter estimated by the fit procedures for a given data set.

Scheme I

$$A \xrightarrow[\text{nucleophile}]{k_0} B \xrightarrow[\Delta V_1]{k_1} C \xrightarrow[\Delta V_2]{k_2} D$$

point, the linkage peptide in C4 was cleaved with trypsin at a time following methylamine addition (20 mM, 37 °C, 17 min) when thioester cleavage was essentially complete while the conformational change was only 35% complete as assessed by the decrease in ANS fluorescence emission. The addition of the enzyme (1% w/w, 45 s) brought about a concomitant decrease in the ANS fluorescence signal to within 10% of the expected equilibrium value (data not shown).

We have attempted to mechanistically analyze the data sets shown in Figure 4 in terms of a scheme (Scheme I) in which the pseudo-first-order chemical modification event was spectroscopically "silent", while subsequent unimolecular conformational processes gave rise to the observed spectroscopic change.

In Scheme I, A represents native C4, B the thioester-cleaved but conformationally unaltered molecule, C an intermediate conformational state, and D the final conformational state. ΔV_1 and ΔV_2 refer to the molar signal change (in volts) occurring in each kinetic phase. The analysis procedure used was essentially the same as that used previously in the kinetic analysis of the nucleophile-induced conformational change in C3 (Isenman et al., 1981). In the present case, however, all steps were made irreversible since in preliminary analyses the fitting procedure consistently made all reverse rate constants

Scheme II

$$A \xrightarrow[\text{nucleophile}]{k_0} B \xrightarrow[\Delta V_1]{k_1} C$$

less than 1% of the values of the forward rate constants. We have, however, encountered a number of problems with the C4 data which did not arise in our previous studies on C3. For example, in the CD kinetics, there apeared to be a very slow, almost linear, change superimposed on the end of the reaction (see Figure 4A) which we believe is probably an artifact arising from the long periods of UV irradiation.[3] If an analysis of these data in terms of the four-species mechanism shown in Scheme I is attempted, the fitting procedure will extrapolate this slow change and predict a total molar signal change which is significantly larger than that observed when the conformational change is allowed to take place in the absence of continuous UV irradiation. We have assumed that the superimposed slow signal increase is linear and have used the slope of a linear regression line drawn through data points from 10 000 s onward (Figure 4A) to subtract away this presumed artifact from the complete data set (see Figure 4B). A good fit of the data could now be obtained by using a three-species mechanism (Scheme II) which has only one spectroscopically detectable conformational change in addition to the spectroscopically silent chemical modification event.

[3] If the sample was intermittently placed on the UV beam, the slow signal increase at long times was greatly diminished; however, insufficient data points could be collected to permit a mechanistic analysis.

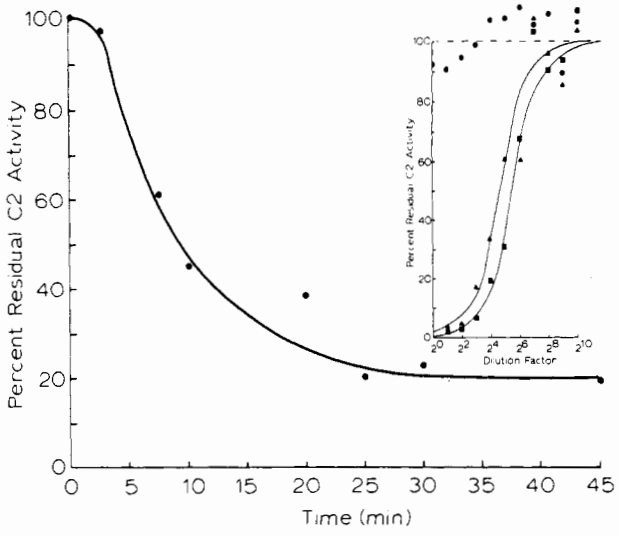

FIGURE 5: Time course appearance of fluid-phase C2 binding activity by C4 treated with methylamine (20 mM, pH 8.0, 37 °C). The times indicated represent the time of sampling of the incubation mixture. The final C4 concentration in the incubation mixture was 2.1×10^{-9} M, and the C2 concentration was 4.7×10^{-10} M. Inset: Concentration dependence of C2 hemolytic inhibition by the fluid phase. C4b (■); $C4(CH_3NH_2)$ (▲); native C4 (●). The initial concentration of C4, C4b, or $C4(CH_3NH_2)$ (i.e., in the 2^0 dilution assay tube) was 2.2×10^{-8} M. For details of the assay system, see Materials and Methods.

The kinetic constants derived from the data set shown in Figure 4B are given in Table II. The half-time of the spectroscopically detectable conformational event (B to C) is about 10.4 min, while the half-time for the chemical modification step (A to B) is approximately 3 min. This latter value is in reasonable accord with the half-time value of 2 min obtained when thioester cleavage was assessed hemolytically.

In the ANS monitoring system (Figure 4C), the fitting algorithm had difficulty in handling the extremely long lag phase, and even a moderately successful fit (see Figure 4C) could only be obtained if one analyzed the data in terms of the four-species mechanism shown in Scheme I and made ΔV_1 negligibly small in the parameter estimates given to the program. The kinetic constants predicted by this mechanism are shown in Table II. The spectroscopically silent portion of the kinetic curve is thus accounted for by both the nucleophilic cleavage event (A to B), for which the fitting algorithm predicts a half-time of 2.5 min, and the subsequent B to C conversion which occurs at a similar rate. The very poor confidence limits associated with these parameters are indicative of the freedom allowed by the program when there is a prolonged period of zero signal change which must be accounted for. Finally the spectroscopically detectable C to D conformational transition has a half-time for completion of about 13.9 min. The differences observed in the kinetics of the methylamine-induced conformational changes in C4 by using the two monitoring systems suggest that the nature of the transition is likely more complex than the minimal schemes which we have employed in our mechanistic analysis. For example, it is possible that the long lag phase observed in the ANS monitoring system reflects the presence of an early kinetic intermediate which gives rise to a signal change of the opposite direction to that observed in the transition to the final conformational state. Similarly, the single detectable conformational change observed in the CD monitoring system may be composed of two or more kinetically unresolved intermediate states.

(3) Kinetics of Acquisition of C4b-like Functional Activity. In experiments using completely transconformed C4-

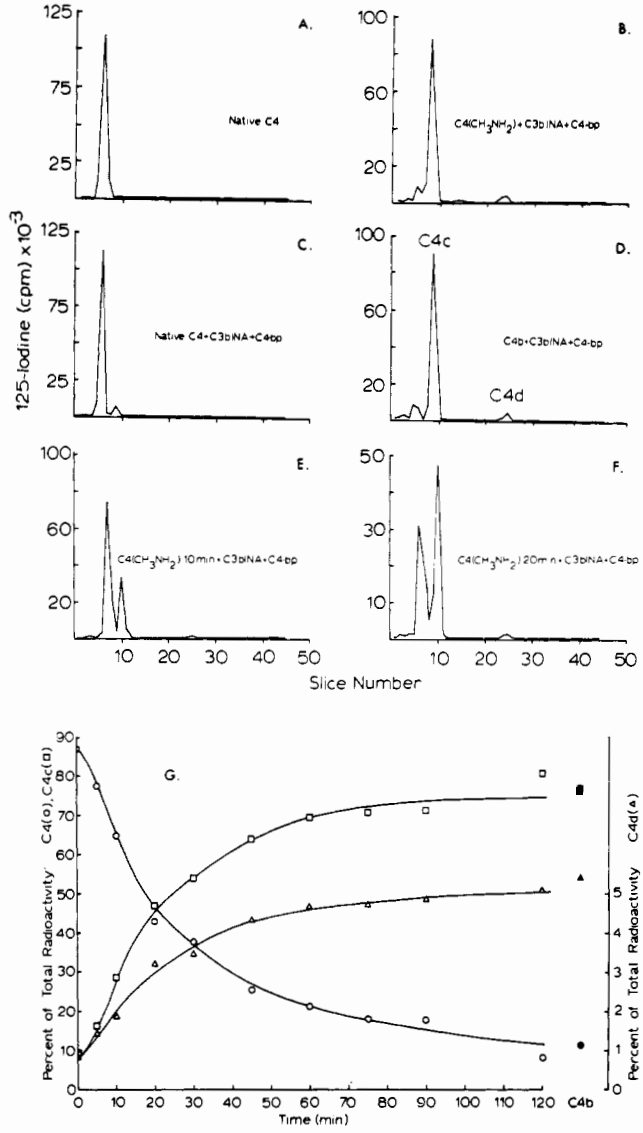

FIGURE 6: Susceptibility to cleavage by C3bINA and C4-bp of $C4(CH_3NH_2)$. (A–D) $NaDodSO_4$–polyacrylamide gel electrophoresis run in the absence of reducing agent of native C4 and of C4, C4-(CH_3NH_2), and C4b (10 μg), each pretreated with C4-bp (10 μg) and C3bINA (2 μg) for 5 min at 37 °C. These digestion conditions were chosen because they produced near-quantitative fragmentation of C4b and $C4(CH_3NH_2)$ within a 5-min pulse as was required for the kinetic experiments shown in panels E–G. (E–G) Time course appearance of susceptibility to cleavage by C3bINA and C4-bp in C4 treated with methylamine (20 mM, pH 8.0, 37 °C). Assay conditions were as described above, and the times indicated refer to the time after methylamine addition when the 5-min pulse with C3bINA and C4-bp was initiated. Panels E and F depict examples of the gels at two intermediate times (10 and 20 min) after methylamine addition. The percentage of the total radioactivity per gel under each peak was used to construct the kinetic plots (open symbols) shown in panel G. The closed symbols shown on the far right depict the percentage of radioactivity in C4c, C4d, and residual C4b when C1s-generated C4b is pulsed with C3bINA and C4-bp as described above.

(CH_3NH_2), we were able to show that this molecule possessed C4b-like functional properties. For example, the experiment shown in the inset to Figure 5 demonstrates that $C4(CH_3NH_2)$ was almost equipotent with C4b in its ability to bind C2 in the fluid phase. Similarly, it can be seen (Figure 6, panels A–D) that $C4(CH_3NH_2)$ is also susceptible to cleavage by C3bINA in the presence of the cofactor C4-bp. When analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, in the absence of reducing agents, the cleaved molecule shows two fragments, one corresponding in molecular weight to C4d

(45 000) and the other being slightly larger than C4c (M_r = 155 000), presumably due to the still attached C4a portion of the molecule. Similar findings on the C4b-like properties of nucleophile-modified C4 were independently obtained by Von Zabern et al. (1981).

The kinetics of the acquisition of the two aforementioned C4b-like properties following the addition of methylamine (20 mM, pH 8.0, 37 °C) to native C4 were investigated, and the results of these experiments are shown in Figures 5 and 6E–G, respectively. In both cases, it is clear that the appearance of the functional activity is slower than the rate of thioester cleavage under the specified conditions. Interestingly, the two activities do not appear simultaneously. Whereas the acquisition of C2 binding activity has a half-time for completion of about 7 min, the rate at which the nucleophile-treated protein becomes susceptible to cleavage by a mixture of C3bINA and C4-bp is considerably slower and requires approximately 20 min for 50% of the protein to become cleavable. This latter time corresponds quite closely to the time at which the ANS fluorescence change is half complete, while the more rapid appearance of a spectroscopically observable change when the CD signal at 262 nm is used as the probe may reflect the presence of an earlier conformational intermediate in which the site responsible for C2 binding has been formed.

Conclusions

The numerous functional activities expressed by the activated form of C4 (C4b) are best explained as arising from proteolytic cleavage induced conformational changes within the molecule. A number of investigations have provided indirect evidence for such conformational transitions. For example, Reboul et al. (1979) have shown that the susceptibility of the constituent chains of C4 to lactoperoxidase-catalyzed radioiodination reflected the hemolytic state of the molecule. Small immunochemical differences between native C4 and C4b have also been noted (Gorski & Müller-Eberhard, 1978; Von Zabern et al., 1981). In the present study, we have provided direct physical evidence for significant conformational alterations in the molecule following proteolytic activation by C1s. As judged by a number of spectroscopic criteria, a virtually identical conformational end state was also produced by nucleophilic scission of the internal thioester bond in C4 in the absence of any proteolysis. Clearly, it is the integrity of this cyclic structure which is the determining factor in maintaining the native conformation of the C4 molecule. The specific proteolytic cleavage in the α chain produced by C1s would therefore appear to have the following two functions. First, it in some way increases the susceptibility of the thioester to nucleophilic or solvolytic scission. Whether this effect is mediated by a peptide cleavage induced increase in the electrophilicity of the reactive carbonyl, or simply by increasing the accessibility of the solvent to this structure, is as yet unknown. Second, only in the proteolytically cleaved molecule does the conformational change occur rapidly. Thus, while the tertiary folding rearrangement is thermodynamically driven by the scission of the thioester bond, kinetic constraints on this process are imposed by the C4a region of the molecule when it remains covalently attached. This kinetic constraint is suggestive of (but does not prove) close contact between the activation peptide and the thioester-containing region of the molecule.

In both its equilibrium and kinetic aspects, the conformational dependence of C4 on the integrity of its thioester bond is analogous to the situation we have observed previously in C3 (Isenman et al., 1981). Whether a similar conformational dependence exists in α_2-macroglobulin, the other known thioester-containing protein (Howard, 1981), remains to be determined.

The C4b-like functional properties of C4(CH_3NH_2) are clearly a manifestation of the molecule having achieved the same conformational end state as in proteolytically activated C4. The ability to monitor the kinetics of functional site appearance following nucleophilic modification has provided a novel approach to the mapping of the various protein interaction sites in the C4 molecule. For example, Gigli et al. (1979) have previously shown that the binding of C4-bp to C4b was antagonistic to the formation of the classical pathway C3 convertase enzyme (C4b2a). While they concluded that this effect was probably due to both an acceleration of C2a dissociation from C4b as well as an inhibition of C2 uptake by this molecule, it could not be determined from their studies whether C4-bp exerted its effect through a steric or allosteric mechanism. Our finding that the appearance of C2 binding activity in C4(CH_3NH_2) substantially precedes in time the molecule's ability to interact with C4-bp, as assessed by it becoming cleavable by C3bINA in the fluid phase (Fujita et al., 1978), suggests that the two sites are distinct and therefore strongly argues in favor of the allosteric mechanism.

Since a thioester-cleaved form of C3 possessing C3b-like functional activity could be formed spontaneously in plasma as a result of water hydrolysis, Pangburn & Müller-Eberhard (1980) have proposed a role for this molecule in the initiation of the alternative complement pathway. The question then arises as to whether the acquisition of C4b-like properties by spontaneously thioester cleaved plasma C4 has any physiologic importance. In order for such a molecule to form a C3 convertase in nonactivated plasma, a protease other than C1s would have to activate the bound C2, and in addition, the inhibitory effect of C4-bp would have to be overcome. We were unable to demonstrate any C3 consumption in Mg^{2+}-EGTA serum brought about by an incubation (37 °C, 60 min) with C4(CH_3NH_2) or C4b at concentrations up to 0.4 mg/mL. This observation may merely reflect the efficiency of the regulatory processes in normal plasma. Since C2 can likely be cleaved by plasma serine proteases other than C1s (e.g., plasmin or kallikrein), if an individual were to be genetically deficient in C4-bp the C4b-like molecules could then accumulate and would probably mediate a consumption of C3 and C2, even if all other complement components and regulatory proteins were produced at their normal levels.

Acknowledgments

We thank Dr. Katherine Siminovitch, Department of Immunology, Toronto Western Hospital, for providing the C4-deficient human serum. We are also grateful to Dr. R. H. Painter of our department for providing the C1s. Finally, we express our appreciation to Janet Young for her excellent technical assistance and to Rose Ann Martino and Kathy Hutton for their secretarial competence.

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